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Epstein-Barr Virus Lytic Infection Is Required for Efficient Production of the Angiogenesis Factor Vascular Endothelial Growth Factor in Lymphoblastoid Cell Lines

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Although Epstein-Barr virus (EBV)-associated malignancies are primarily composed of cells with one of the latent forms of EBV infection, a small subset of tumor cells containing the lytic form of infection is often observed. Whether the rare lytically infected tumor cells contribute to the growth of the latently infected tumor cells is unclear. Here we have investigated whether the lytically infected subset of early-passage lymphoblastoid cell lines (LCLs) could potentially contribute to tumor growth through the production of angiogenesis factors. We demonstrate that supernatants from early-passage LCLs infected with BZLF1-deleted virus (Z-KO LCLs) are highly impaired in promoting endothelial cell tube formation in vitro compared to wild-type (WT) LCL supernatants. Furthermore, expression of the BZLF1 gene product in *trans* in Z-KO LCLs restored angiogenic capacity. The supernatants of Z-KO LCLs, as well as supernatants from LCLs derived with a BRLF1-deleted virus (R-KO LCLs), contained much less vascular endothelial growth factor (VEGF) in comparison to WT LCLs. BZLF1 gene expression in Z-KO LCLs restored the VEGF level in the supernatant. However, the cellular level of VEGF mRNA was similar in Z-KO, R-KO, and WT LCLs, suggesting that lytic infection may enhance VEGF translation or secretion. Interestingly, a portion of the vasculature in LCL tumors in SCID mice was derived from the human LCLs. These results suggest that lytically infected cells may contribute to the growth of EBV-associated malignancies by enhancing angiogenesis. In addition, as VEGF is a pleiotropic factor with effects other than angiogenesis, lytically induced VEGF secretion may potentially contribute to viral pathogenesis.

Epstein-Barr virus (EBV), a ubiquitous human herpes virus, can exist in either a latent or lytic state with regard to viral gene expression. In the latent forms of infection, a limited subset of viral genes is expressed, and the virus is replicated using the host cell DNA polymerase (53). The EBV proteins known to be important for cellular transformation are expressed during the type II and type III forms of latent infection (28, 33, 58, 63). In lytic infection, widespread viral gene expression occurs, resulting in replication of the virus by viral DNA polymerase, release of infectious virus, and death of the host cell (53). Entry into lytic cycle is triggered by expression of the two immediate-early genes of EBV, BZLF1 and BRLF1. The BZLF1 and BRLF1 gene products function as transcription factors with the capacity to activate both viral and cellular promoters (9, 37, 39, 53).

EBV is associated with numerous malignancies of both B-cell and epithelial cell origin. EBV-associated malignancies are primarily infected with one of the latent forms of EBV, although a small subset of lytically infected cells is commonly detected in biopsies of EBV-positive malignancies (11, 42, 70). BZLF1-deleted (Z-KO) and BRLF1-deleted (R-KO) viruses are unable to replicate lytically but can immortalize primary B cells in vitro with similar efficiency as the wild-type (WT) virus (17). However, a potential role for lytically infected cells in promoting tumor growth in vivo was suggested by our recent finding that early-passage lymphoblastoid cell lines (LCLs) derived with either Z-KO or R-KO EBV (Z-KO or R-KO LCLs, respectively) were impaired for growth in SCID mice in comparison to LCLs infected with WT EBV (WT LCLs) (26). Restoration of the capacity of Z-KO LCLs to enter the lytic cycle (accomplished by introducing a BZLF1 expression vector in *trans*) resulted in enhanced growth of Z-KO LCLs in SCID mice. Furthermore, LCLs derived from the Z-KO and R-KO viruses expressed significantly less interleukin-6 (IL-6), cellular IL-10, and viral IL-10 in vitro in comparison to WT LCLs, suggesting that the in vivo tumorigenesis defect may be due at least partly to decreased secretion of paracrine B-cell growth factors.

Angiogenesis, or the formation of new blood vessels, is a critical event for the efficient growth of both B-cell and epi-

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thelial cell malignancies, as all cells must be located within ~100 μm of a capillary in order to survive (8). In addition, angiogenesis provides tumor cells with access to the host vasculature, thereby allowing dissemination and metastasis. Angiogenesis is a tightly regulated process in which the relative balance between proangiogenic factors and antiangiogenic factors determines whether new vessels are formed. Tumors induce angiogenesis by disrupting this balance, often by upregulating secretion of proangiogenic factors such as vascular endothelial growth factor (VEGF) (8).

Virus-induced upregulation of angiogenesis factors in virally mediated malignancies may play a significant role in tumor progression. In the case of Kaposi's sarcoma-associated herpes virus, a close relative of EBV, multiple viral lytic proteins are capable of inducing upregulation of VEGF (3, 6, 66). The capacity of VEGF to induce angiogenesis is thought to contribute to the development of Kaposi's sarcoma (6), a highly vascularized lesion, while the ability of VEGF to increase vascular permeability may play a role in the pathogenesis of Kaposi's sarcoma-associated herpes virus-associated primary effusion lymphoma (4, 5). Angiogenesis is also thought to be critically important for the growth of the EBV-associated malignancies nasopharyngeal carcinoma (NPC) and Burkitt's lymphoma (BL), as angiogenesis inhibitors are able to inhibit tumor growth in mouse models of either malignancy (48, 50, 71). The viral latent protein LMP1, which is expressed in some cases of NPC, may play a role by inducing expression of angiogenic factors such as VEGF, IL-8, and IL-6 in latently infected tumor cells (15, 16, 43, 72). The contribution of EBV lytic proteins to the regulation of tumor angiogenesis is largely unknown.

In this paper we have examined whether lytic-defective Z-KO LCLs are impaired in mediating angiogenesis in comparison to WT LCLs. Somewhat surprisingly, especially considering that only a few percent of WT LCLs have the lytic form of viral infection, the supernatant of Z-KO LCLs from two separate donors had significantly lower angiogenic activity than the supernatant from WT LCLs. Furthermore, BZLF1 expression in *trans* restored angiogenic activity in the Z-KO LCL supernatants. In comparison to WT LCLs, Z-KO and R-KO LCLs secreted much lower amounts of the angiogenic factor VEGF in vitro, and reintroduction of BZLF1 expression in the Z-KO LCLs resulted in increased secretion of VEGF. The difference in VEGF secretion in the lytic-defective versus WT LCLs was not due to differences in transcription of VEGF, since the WT and Z-KO LCLs expressed similar levels of VEGF mRNA. Instead, one or more lytic EBV proteins presumably increase translation and/or secretion of VEGF. These results suggest that in early-passage LCLs, angiogenic factors are primarily derived from a small number of lytically infected cells. The decreased secretion of angiogenesis factors in early-passage lytic-defective LCLs, coupled with their known defect in forming tumors in vivo, suggests that lytic induction of angiogenesis factors such as VEGF may play a significant role in growth of some EBV-associated tumors.

MATERIALS AND METHODS

EBV wild-type, Z-KO, and R-KO viruses and cell lines. 293 cells infected with the R-KO virus, Z-KO virus, and WT virus have been described previously (17). In the R-KO virus, nucleotides 103638 to 105083 (strain B95.8 coordinates, accession number V01555) within the BRLF1 gene were removed via insertion of a tetracycline resistance cassette. In the Z-KO virus, nucleotides 102389 to 103388 (B95.8 coordinates) within the BZLF1 gene were removed via insertion of a kanamycin resistance cassette. The R-KO, Z-KO, and WT viruses also encode enhanced green fluorescent protein and a hygromycin B resistance gene (both cloned into the B95.8 deletion of EBV where the second copy of oriLyt normally resides). LCLs containing the WT, Z-KO, and R-KO viruses were generated as described previously (25), using the supernatant of BZLF1- or BRLF1-transfected 293 cells infected with Z-KO or R-KO virus, respectively, as a source of virus and peripheral blood leukocytes derived from several different anonymous donors (obtained from the American Red Cross). LCLs were maintained in RPMI medium supplemented with 10% fetal bovine serum and antibiotics. Z-KO and R-KO LCLs were verified to be $BZLF1^-$ and $BRLF1^-$ via PCR with BZLF1- and BRLF1-specific primers.

Generation of Z-KO LCLs expressing BZLF1. pREP9-Zp-Z (which contains the BZLF1 gene driven by its own promoter) was generated by cloning the 1.8-kb BamHI Z EBV fragment (B95.8 genome) into the BamHI site of a modified pREP9 vector (Invitrogen) in which the Rous sarcoma virus promoter had been removed and a blasticidin resistance cassette was inserted. The pREP9 plasmid contains the EBV *oriP* element and hence can be stably maintained as an episome in EBNA-1-expressing LCLs. Stable Z-KO LCL lines containing either the vector control (Z-KO-vector LCLs) or the pREP9-ZpZ expression vector were generated using a nucleoporator (Amaxa) as previously described (26).

Matrigel in vitro HDMEC tube formation assay. Human dermal microvascular endothelial cells (HDMECs) were maintained in EGM-2 medium supplemented with 5% fetal bovine serum. Matrigel (125 μl; growth factor reduced), after being thawed on ice, was plated in eight-well chamber slides. These slides were then incubated at 37°C for 30 min to allow the Matrigel to polymerize. HDMECs (400 μl; 4×10^4 cells/ml in EBM-2 medium without serum) were added to each well and treated with 40 μl of culture supernatant from LCLs (1×10^6 cells cultured in 1 ml of serum-free medium for 24 h). VEGF (50 ng/ml) was used as positive control, and medium alone was used as a negative control. The chamber slides were then incubated for 16 to 18 h at 37°C in a 5% CO_2 humidified atmosphere. At the end of incubation, the culture medium was very carefully aspirated off the Matrigel surface, and the cells were fixed with methanol and stained with Diff-Quick solution II. Each chamber was photographed using an inverted microscope with a digital camera (Leica DMIRB, Germany) and total tube area in each chamber was quantified using Metamorph software (Universal Imaging Corporation, Downingtown, PA) and expressed as an angiogenic score. *P* values were calculated using an Excel *t* test.

In vivo angiogenesis studies. LCL tumors generated in SCID mice injected subcutaneously with 5×10^6 WT or Z-KO LCLs from the same donor (26) were harvested once they reached ~4 mm in diameter, fixed overnight in 10% neutral buffered formalin, and embedded in paraffin. Hematoxylin- and eosin-stained sections were examined under high power, and the number of vessels per five high-powered fields was counted and averaged. For identification of human-derived $CD31^+$ cells, tumor sections were deparaffinized and rehydrated. Following epitope retrieval, slides were stained using a human specific CD31 antibody (clone JC/70A; Ventana) on a BenchMark instrument (Ventana Medical Systems, Inc).

Immunoblotting. Supernatants of LCLs growing for 2 days in serum-free medium were concentrated ~50-fold using ultrafiltration with Centricon columns (Millipore, Billerica, MA). Equal amounts (micrograms) of concentrated supernatants were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted using standard procedures with the following primary antibodies: polyclonal rabbit anti-VEGF-A (1:500; Santa Cruz Biotechnology) and polyclonal rabbit anti-basic fibroblast growth factor (bFGF) (1:500; Santa Cruz Biotechnology). Protein extracts from the cellular pellets collected at the same time as the supernatants were also immunoblotted as described above using the anti-VEGF-A antibody.

Reverse-transcription PCR (RT-PCR) analysis. LCLs were grown in serum-free medium for 24 h, and total RNA was harvested using a QIAGEN RNeasy Kit according to the manufacturer's instructions. One microgram of RNA was reverse transcribed using a Promega Reverse Transcription system according to the manufacturer's instructions. PCR was then performed using primers specific for VEGF (7) and B_2-microglobulin (41) using different dilutions of cDNA.

RESULTS

Z-KO LCL supernatants are less angiogenic than WT LCL supernatants in vitro. To determine whether lytic infection contributes to tumor angiogenesis, we utilized early-passage Z-KO

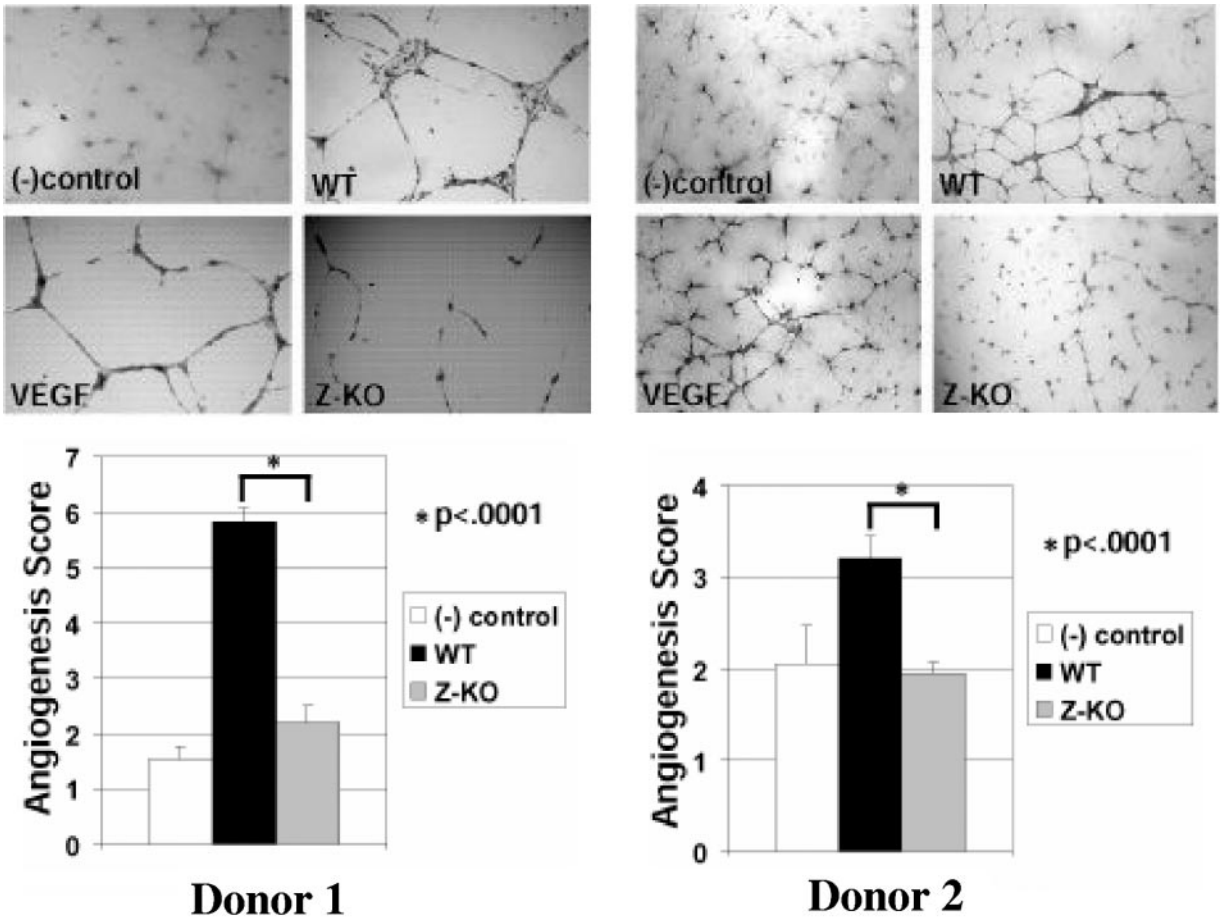

FIG. 1. Z-KO LCL supernatants are less angiogenic than WT LCL supernatants in vitro. Supernatants from WT LCLs (WT) or Z-KO LCLs (Z-KO) growing in vitro were tested for their ability to induce tube or vessel formation of HDMECs in vitro. VEGF, HDMECs treated with the known angiogenic factor VEGF; (−) control, HDMECs treated with medium alone. Photomicrographs of a representative area of endothelial tube formation within a chamber for each sample from donor 1 and donor 2 are shown. Quantification of the total tube area per chamber formed by HDMECs treated with medium alone (control), medium from WT LCLs, or medium from Z-KO LCLs expressed as an angiogenic score (*y* axis) is shown for each donor. Data represent the means of three independent experiments, each done in triplicate. Error bars indicate standard errors of the mean. The results of LCLs derived from two different donors are shown.

LCLs, which we recently discovered have a tumorigenesis defect in SCID mice (26). Z-KO LCLs are infected with a recombinant EBV in which the BZLF1 immediate-early gene has been deleted via insertional mutagenesis (17). The Z-KO virus cannot replicate lytically unless BZLF1 is supplied in *trans* (17) and, hence, represents a lytic-defective virus. Supernatants from early-passage Z-KO or WT LCLs growing in vitro (derived from the same donor) were tested for their ability to induce endothelial tube formation in a Matrigel in vitro angiogenesis assay (Fig. 1). WT LCL supernatants, as well as the known angiogenic factor VEGF, were able to induce significant endothelial tube formation. In sharp contrast, Z-KO LCL supernatants induced minimal tube formation. Quantification of tube formation revealed that WT LCL supernatants contained approximately threefold more angiogenic activity than Z-KO LCL supernatants (Fig. 1, right panel) ($P < 0.0001$).

Given that the donor origin of LCLs can influence their behavior both in vitro and in vivo (14, 30, 47), we next determined if Z-KO LCLs derived from a second donor were also defective in their capacity to secrete angiogenesis factors in comparison to the corresponding WT LCLs. Z-KO LCLs from the second donor also secreted lower levels of angiogenesis factors in comparison to the corresponding WT LCLs (Fig. 1), indicating that the angiogenesis defect of Z-KO LCLs is not donor specific. The decreased angiogenic activity of Z-KO supernatants surprisingly suggests that the majority of secreted angiogenesis factors in early-passage LCLs is derived from the rare, lytically infected cells.

Restoring expression of BZLF1 in *trans* increases the angiogenic capacity of Z-KO LCLs. Previous studies have demonstrated that Z-KO LCLs have a similar viral latency profile to WT LCLs (17, 26). To further verify that the difference between WT and Z-KO LCLs with regard to angiogenesis was solely due to a lack of lytic gene expression, we created Z-KO LCLs stably carrying either an *oriP*-replicated episomal control vector (Z-KO-vector LCLs) or the same vector containing the BZLF1 gene driven by the endogenous BZLF1 promoter (Z-KO-ZpZ LCLs). Z-KO-ZpZ LCLs behaved similarly to WT LCLs in their capacity to be induced into the lytic cycle in vitro (data not shown); furthermore, tumors formed in SCID

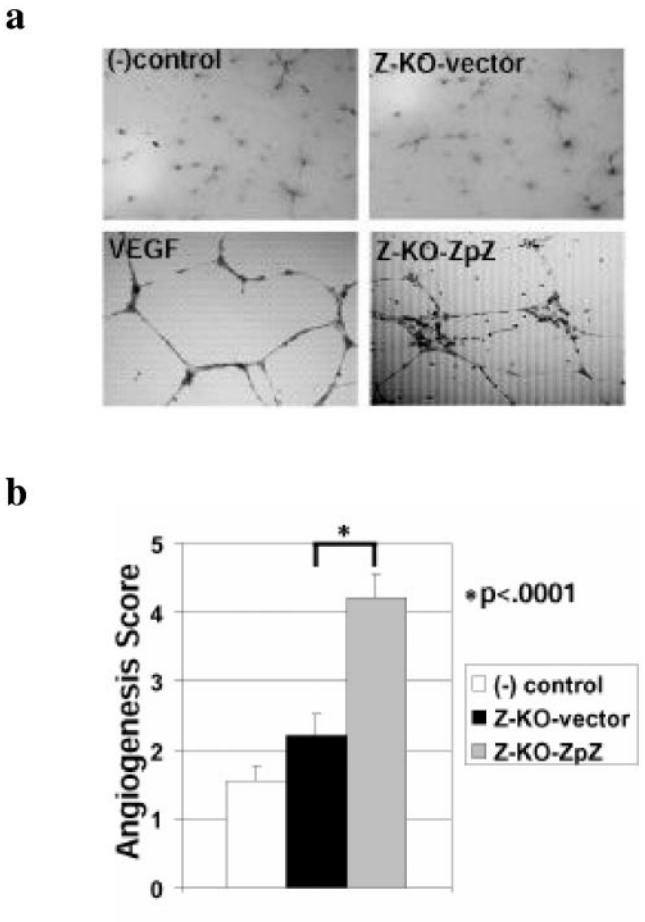

FIG. 2. Restoring the expression of BZLF1 in *trans* increases the angiogenic capacity of Z-KO LCLs. (a) Supernatants from Z-KO LCLs carrying a control vector (Z-KO-vector) or carrying a BZLF1 expression vector driven by the authentic BZLF1 promoter (Z-KO-ZpZ) were tested for their ability to induce tube and vessel formation of HDMECs in vitro. VEGF, HDMECs treated with the known angiogenic factor VEGF; (−) control, HDMECs treated with medium alone. Photomicrographs of a representative area of endothelial tube formation within a chamber for each sample are shown. (b) Quantification of the total tube area per chamber formed by HDMECs treated with medium alone (control), medium from Z-KO LCLs carrying a control vector (Z-KO-vector), or medium from Z-KO LCLs carrying a BZLF1 expression vector (Z-KO-ZpZ), expressed as an angiogenic score (*y* axis). Data represent the means of three independent experiments, each done in quadruplicate. Error bars indicate standard errors of the mean.

mice by Z-KO-ZpZ LCLs expressed viral lytic proteins in a few percent of cells at a comparable level to WT LCL tumors (26). As depicted in Fig. 2a, the supernatants from Z-KO-ZpZ LCLs possessed approximately twofold higher angiogenic activity than the Z-KO-vector supernatants, and this difference was statistically significant ($P < 0.0001$) (Fig. 2b). The increased angiogenic capacity of Z-KO-ZpZ supernatants indicates that restoration of BZLF1 expression increases secretion of angiogenic factors.

Examination of angiogenesis in vivo. In order to determine whether the in vitro angiogenesis defect in the Z-KO LCLs could also be demonstrated in vivo, we analyzed tumors arising in SCID mice following injection with either Z-KO or WT LCLs. The degree of angiogenesis was not significantly different between WT and Z-KO LCL tumors as determined by vessel counts of hematoxylin- and eosin-stained tumor sections. The lack of an in vivo angiogenesis difference may simply reflect the possibility that the tumors were harvested at a point where an angiogenesis difference was minimized or no longer apparent (see Discussion).

A recent report suggests that the vasculature within human posttransplant lymphoproliferative disease (PTLD) is, unexpectedly, partially derived from lymphoma cells (61). The LCL tumor vasculature in SCID mice can be stained with an antibody specific to the mouse form of CD31 (a marker routinely used for detection of endothelial cells), and therefore at least a portion of the vasculature in this animal model for PTLD is derived from murine endothelial cells (69). To determine if the vasculature within LCL tumors in SCID mice is also partially derived from the LCLs themselves, we stained the tumors using an antibody specific for the human form of CD31 (Fig. 3). Notably, some tumor vessels (Fig. 3a, arrow) as well as individual LCLs (Fig. 3b, arrow) were strongly positive for human CD31. While CD31 can be detected in lymphomas and in some normal B-cell subsets (59), the human origin of the tumor vasculature suggests that LCLs may directly contribute to the formation of tumor vessels in SCID mice. However, we did not detect a consistent difference in the degree of human CD31 staining in either LCLs or the tumor vasculature between WT- and Z-KO LCL-derived tumors, suggesting that lytic infection is not required for the ability of LCLs to become endothelial cells.

Lytic-defective LCLs secrete less VEGF than WT LCLs in vitro. Of the many positive regulators of angiogenesis, VEGF represents the most potent angiogenic factor characterized to date (18). To determine if the angiogenesis defect of Z-KO LCLs was a consequence of decreased VEGF secretion, we performed Western blot analysis of supernatants harvested from serum-starved WT and Z-KO LCLs growing in vitro. As depicted in Fig. 4a, Z-KO LCLs secreted markedly lower levels of VEGF than WT LCLs, suggesting that lytically infected cells are the major source of VEGF secretion. To verify that the defect in VEGF secretion was due to a defect in lytic infection, we also examined the supernatants from LCLs derived with the lytic-defective R-KO virus. Similar to the Z-KO LCLs, R-KO LCLs are also impaired for tumorigenesis in vivo while exhibiting a similar latency profile to WT LCLs (26). In comparison to WT LCLs, R-KO LCLs (from the same donor) also exhibited decreased secretion of VEGF (Fig. 4a). The decreased secretion of VEGF in the lytic-defective LCLs did not reflect a nonspecific defect in transcription, translation, or secretion, as equivalent amounts of another well-known angiogenic factor, bFGF, were secreted into the supernatants of WT, Z-KO, and R-KO LCLs (Fig. 4a). Finally, to further demonstrate that a defect in lytic gene expression was responsible for decreased VEGF secretion, we examined supernatants from Z-KO LCLs stably expressing BZLF1 in *trans* (under the control of its natural promoter) (26). As demonstrated in Fig. 4b, Z-KO LCLs carrying the BZLF1 expression vector (Z-KO-ZpZ) secreted higher levels of VEGF than the corresponding Z-KO LCLs carrying the control vector (Z-KO-vector), again suggesting that lytic infection contributes to the release of VEGF.

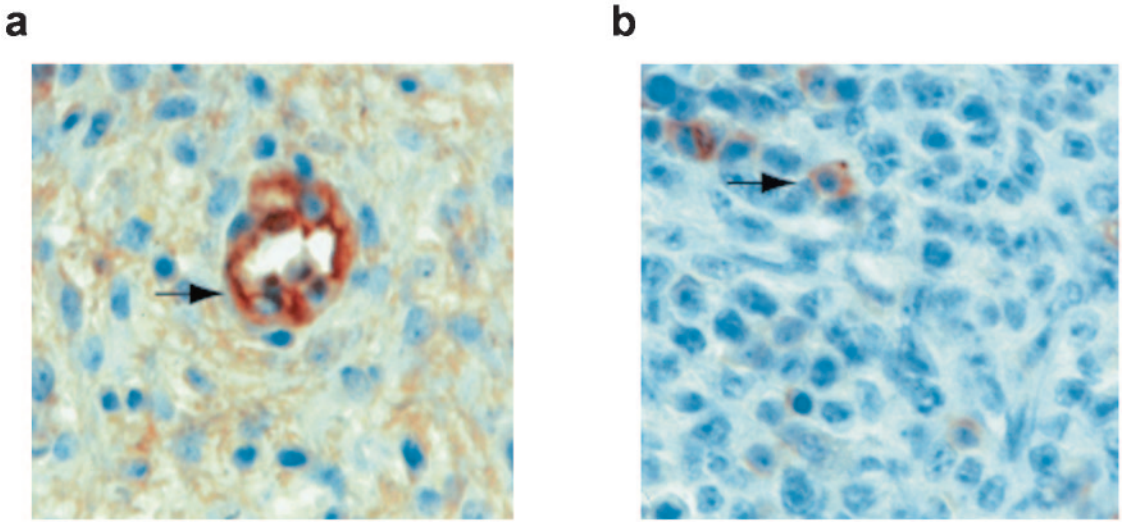

FIG. 3. Examination of angiogenesis in vivo. Z-KO LCL tumors were stained using an antibody specific for human CD31. Examples of tumor vessels (a) and individual LCLs (b) staining positive for human CD31 are indicated by arrows. Similar results were obtained in WT LCL tumors (data not shown).

Lytic infection does not regulate VEGF at the level of transcription. VEGF expression is intricately regulated through both transcriptional and posttranscriptional mechanisms (46). For example, in response to certain stimuli, the cell may upregulate transcription of VEGF, increase stability of the VEGF mRNA, or enhance translational initiation of VEGF transcripts. To determine if the increased secretion of VEGF observed in WT LCLs is due to an upregulation of VEGF transcription, we analyzed VEGF transcript levels using semiquantitative RT-PCR. WT, Z-KO, and R-KO LCLs all expressed similar levels of the various VEGF transcripts (Fig. 5a), suggesting that increased secretion of VEGF in WT LCLs was not due to enhanced VEGF transcription. Furthermore, although reintroduction of BZLF1 expression in the Z-KO LCLs resulted in increased secretion of VEGF, no increase in the level of VEGF transcripts was observed in Z-KO LCLs carrying a BZLF1 expression vector (Fig. 5b), again suggesting that lytic infection enhances VEGF expression/secretion via a posttranscriptional mechanism.

Lytic-defective and WT LCLs have similar amounts of intracellular VEGF protein. Immunoblot analysis was performed on cellular extracts to determine if WT LCLs also have a

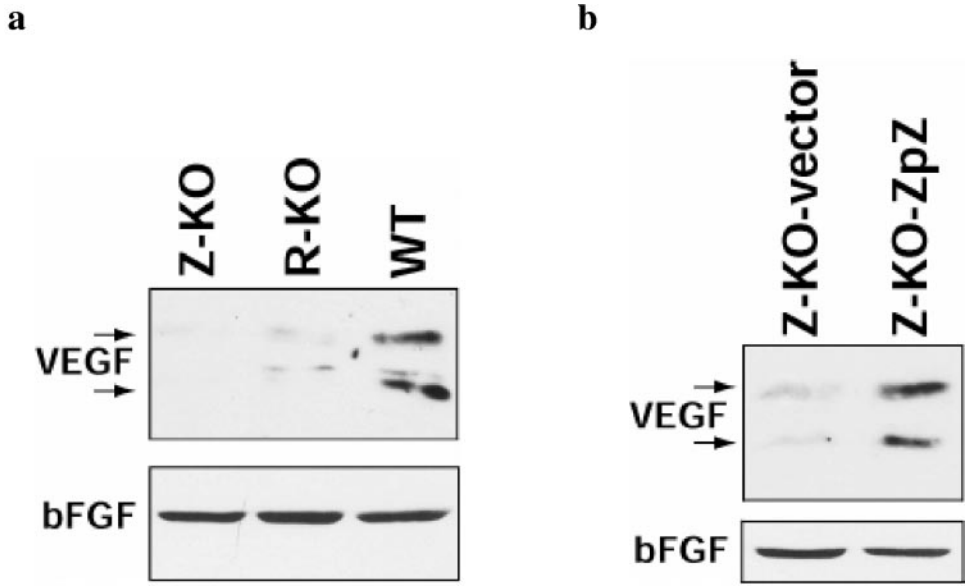

FIG. 4. Lytic-defective LCLs secrete less VEGF than WT LCLs in vitro. (a) Supernatants from WT LCLs (WT), Z-KO LCLs (Z-KO), or R-KO LCLs (R-KO) growing in vitro were concentrated and immunoblotted using either VEGF-A- or bFGF-specific antibodies. Arrows indicate different forms of VEGF generated by alternative splicing. (b) Supernatants from Z-KO LCLs carrying a BZLF1 expression vector (Z-KO-ZpZ) and Z-KO LCLs carrying the control vector (Z-KO-vector) were concentrated and immunoblotted using a VEGF-A specific antibody.

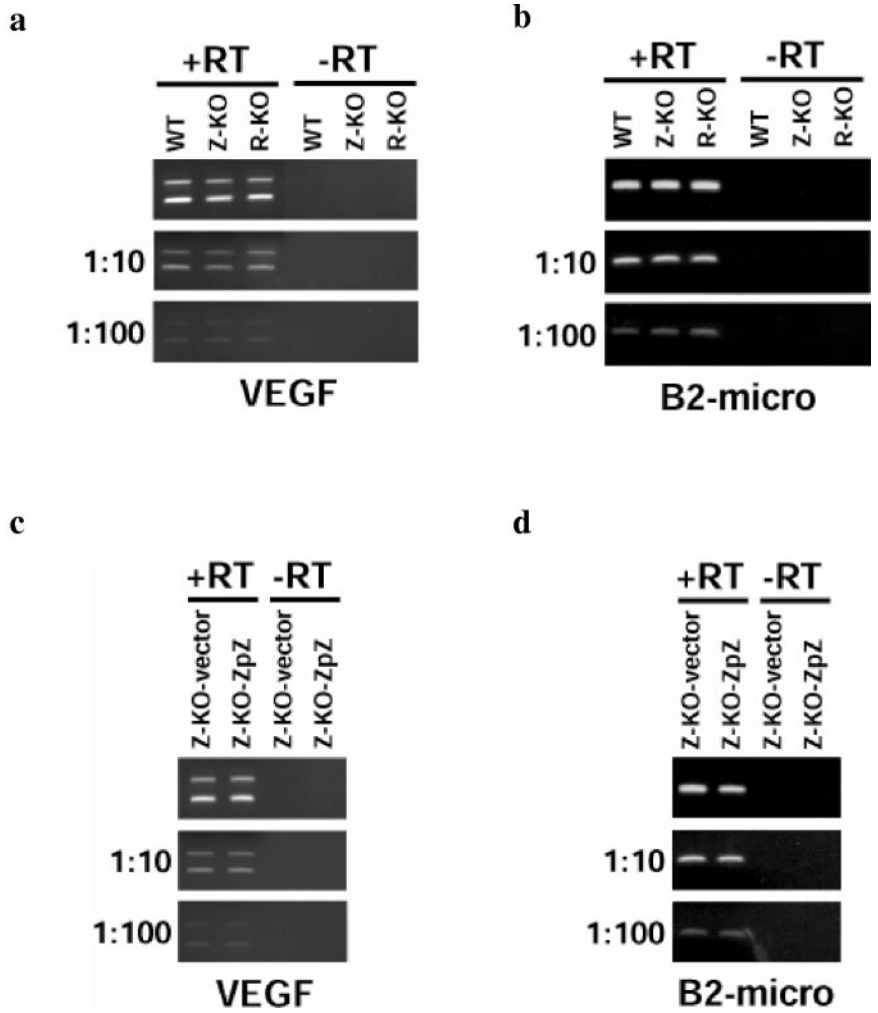

FIG. 5. Lytic infection does not regulate VEGF at the level of transcription. A comparison of VEGF (a) or B_2-microglobulin (B2-micro) (b) transcription using RT-PCR in WT, Z-KO, and R-KO LCLs growing in vitro is shown. VEGF transcripts are alternatively spliced, leading to multiple RT-PCR products. Different dilutions of starting cDNA amounts are shown to ensure linearity. A comparison of VEGF (c) or B_2-microglobulin (B2-micro) (d) transcription using RT-PCR in Z-KO LCLs carrying a control vector (Z-KO-vector) or a BZLF1 expression vector (Z-KO-ZpZ) is shown. Different dilutions of starting cDNA amounts are shown to ensure linearity.

higher level of intracellular VEGF protein than the lytic-defective LCLs. As shown in Fig. 6a the amount of intracellular VEGF protein was similar in the WT LCLs and the Z-KO and R-KO LCLs. Furthermore, restoration of BZLF1 expression in the Z-KO LCLs did not increase the amount of intracellular VEGF (Fig. 6b). Since the total level (intracellular plus extracellular) of VEGF is clearly greater in the WT versus lytic-defective cells, while the amount of intracellular VEGF is similar, these results suggest that lytic infection not only increases the total amount of VEGF but also enhances its secretion.

DISCUSSION

EBV is strongly associated with several malignancies of both B-cell and epithelial origin, and the presence of the virus is believed to contribute to the growth of tumors in vivo (52). Although the majority of cells within an EBV-associated malignancy are latently infected, small subsets of lytically infected cells are frequently detected in several different malignancies (11, 40, 42, 70). A role for lytic infection in malignancy was recently demonstrated by our finding that early-passage LCLs infected with lytic-defective viruses (Z-KO and R-KO LCLs) were impaired for growth in SCID mice (26). Furthermore, Z-KO and R-KO LCLs secreted lower levels of important paracrine B-cell growth factors in vitro, suggesting that lytic infection may contribute to the formation of EBV-associated lymphomas via release of paracrine factors (26).

In this paper we demonstrate that lytic infection in early-passage LCLs also induces angiogenesis, suggesting another potential mechanism by which lytic infection may contribute to malignancy. Supernatants from WT LCLs were more angiogenic than supernatants from Z-KO LCLs, and this effect was not dependent on the donor origin of the LCLs. Introduction of a BZLF1 expression vector into Z-KO LCLs, which restores the capacity of Z-KO LCLs to enter lytic cycle (26), enhanced the angiogenic activity of Z-KO LCL supernatants, confirming that lytic infection in LCLs contributes to release of

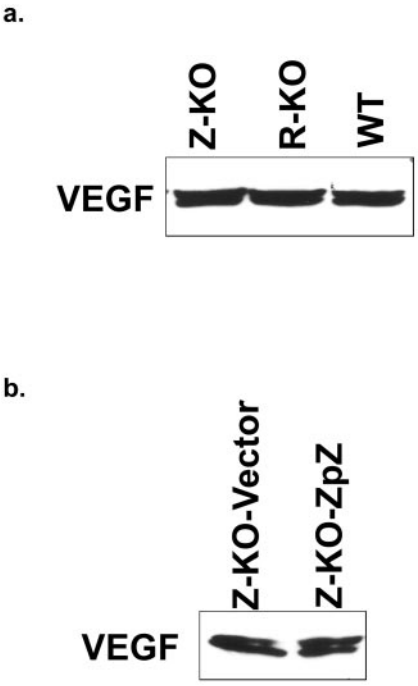

FIG. 6. Lytic-defective LCLs have a similar amount of intracellular VEGF as WT LCLs in vitro. (a) Cellular extracts from WT LCLs (WT), Z-KO LCLs (Z-KO), or R-KO LCLs (R-KO) growing in vitro were immunoblotted using the VEGF-A-specific antibodies. (b) Cellular extracts from Z-KO LCLs carrying a BZLF1 expression vector (Z-KO-ZpZ) and Z-KO LCLs carrying the control vector (Z-KO-vector) were immunoblotted using a VEGF-A-specific antibody.

angiogenesis factors. As growth of LCL tumors in SCID mice is characterized by abundant angiogenesis (69) and angiogenesis inhibitors prevent growth of lymphoma cells in mice (48), the impaired capacity of Z-KO LCLs to secrete angiogenesis factors may contribute to their impaired growth in SCID mice.

Despite the readily apparent in vitro angiogenesis defect of the Z-KO LCLs, we were not able to detect a striking difference in angiogenesis of WT or Z-KO LCLs tumors in vivo. However, it should be noted that tumors were harvested once they had reached a certain size, and since the Z-KO LCLs grow at a significantly slower rate than WT LCLs in mice (26), the Z-KO LCL tumors were harvested at a later time point than the corresponding WT LCL tumors. Hence, by the time the Z-KO LCL tumors reach harvest size (~4 mm in diameter), they might have already overcome an initial angiogenesis defect (e.g., via genetic or epigenetic changes) that hinders growth during the early stages of tumorigenesis. Indeed, as angiogenesis is required after a cell is located >100 μm from an existing vessel (8), it seems likely that Z-KO LCL tumors would have likely overcome any angiogenesis defect in order to reach a size of 4 mm. Surprisingly, both Z-KO and WT LCL tumors exhibited human CD31 staining of the tumor vasculature, suggesting that at least a portion of the tumor vasculature is derived from human LCLs as opposed to mouse endothelial cells. A recent report indicates that the vascular cells within different human B-cell lymphomas share the same specific cytogenetic aberrations as the lymphoma cells, suggesting that the tumor vasculature can be directly derived from the lymphoma cells themselves (61). Interestingly, as lymphoma cells are known to express various degrees of CD31 (59) and hypoxia can regulate differentiation of malignant cells (23, 29), the possibility exists that LCLs could switch to an endothelial cell phenotype in vivo under conditions where angiogenesis is needed.

VEGF represents the most potent angiogenic factor characterized to date and is frequently expressed by human B-cell lymphoma cells in vitro and in vivo (19, 20, 24, 64). Both Z-KO and R-KO LCLs secreted substantially lower levels of VEGF than WT LCLs in vitro, suggesting that lytic infection induces VEGF secretion. Furthermore, introduction of a BZLF1 expression vector into Z-KO LCLs enhanced secretion of VEGF. As angiogenesis is a key requirement for lymphoma growth, the impaired growth of Z-KO LCL tumors may be partly a consequence of decreased angiogenesis due to insufficient VEGF secretion. Decreased secretion of VEGF may also directly inhibit the growth of Z-KO LCLs in vivo, as VEGF can function as an autocrine growth factor for lymphoma cells (65). It should also be noted that VEGF stimulation of tumor stromal cells may result in secretion of IL-6 (12), a potent B-cell growth factor that enhances LCL growth in vivo (13, 62).

In addition to its potential effects during malignancy, lytic-induced VEGF secretion may also influence viral pathogenesis during normal infection. VEGF can functionally inhibit dendritic cell maturation (21), and thus induction of VEGF secretion may assist the virus in lessening the host response during lytic infection. VEGF also possesses antiapoptotic effects (20, 22, 32, 35, 36, 44, 45, 65) and may prolong survival of the host cell during lytic infection. Finally, secretion of VEGF can recruit monocytes (10), a cell type capable of being infected with EBV that has been proposed to serve as a vehicle for viral dissemination (55, 56).

The regulation of VEGF is complex and involves both transcriptional and posttranscriptional mechanisms (46). WT, Z-KO, and R-KO LCLs expressed similar levels of VEGF transcripts, suggesting that the decreased VEGF secretion in lytic-defective LCLs is due to a difference in posttranscriptional regulation. Translation of VEGF mRNA is regulated in a cap-dependent manner by eIF-4E (34), an initiation factor that is activated by stress kinase signaling (51, 67, 68). Interestingly, both the BZLF1 and BRLF1 gene products activate stress kinases (1), suggesting a possible mechanism for enhanced VEGF translation during the lytic viral cycle. VEGF translation is also regulated in a cap-independent manner via two internal ribosome entry site elements present in the 5′ untranslated region of VEGF transcripts (2, 27, 60). Similar to eIF-4E-mediated translation, internal ribosome entry site-mediated translation of some genes can also be positively regulated by stress kinases (57), suggesting yet another mechanism for lytic infection to increase VEGF translation. Our results also suggest the possibility that lytic EBV infection enhances the secretion of VEGF, as the level of extracellular VEGF was clearly increased to a greater degree than the amount of intracellular VEGF in lytically infected LCLs.

It is also possible that angiogenesis factors in addition to VEGF contribute to the increased angiogenicity of the WT LCL supernatants. Although VEGF is the most potent and well-characterized angiogenesis factor to date, many other positive regulators of angiogenesis are known, and the possi-

bility exists that lytic infection induces additional positive regulators of angiogenesis. It should also be noted that EBV has the capacity to infect and replicate within endothelial cells in vitro (31); furthermore, binding of the EBV virion to endothelial cells induces secretion of IL-6 (31), another positive regulator of angiogenesis. Hence, it is possible that lytic infection also contributes to angiogenesis by direct interaction with or infection of endothelial cells by EBV virions.

The angiogenesis studies presented in this paper were all performed using LCLs. LCLs represent a suitable model for EBV-associated PTLD, as injection of LCLs into SCID mice results in lymphomas similar to PTLD in regard to viral gene expression and histology (52, 54). Whether or not lytic infection contributes to angiogenesis in other types or models of EBV malignancy is currently unknown. Latent infection has been proposed to contribute to NPC angiogenesis via LMP1-mediated secretion of the angiogenesis factors VEGF, IL-8, and matrix metalloproteinases (MMPs) (38, 43, 72). Since up to 65% of NPC biopsies exhibit no evidence of LMP1 protein expression (52), lytic infection may be important for the induction of angiogenesis in the absence of LMP1. Indeed, BZLF1 can induce the angiogenesis factors MMP-1/MMP-9 in NPC cells (38, 73) as well as transforming growth factor β(9) in other epithelial cell lines. BL, another EBV-associated B-cell malignancy, frequently exhibits small subsets of lytically infected cells (70) and is dependent on angiogenesis for successful tumor growth in mouse models (48, 49). As BL does not express LMP1, lytic infection may be important for angiogenesis and successful tumor growth in this context.

In conclusion, we demonstrate an unexpected and novel contribution of lytic EBV infection in angiogenesis. In the future it will be important to determine which lytic EBV protein(s) contributes to VEGF induction and the precise mechanism for this posttranscriptional effect. Our demonstration that lytic infection in LCLs contributes to VEGF release has important implications not only for angiogenesis in EBV-associated tumors but also for understanding viral pathogenesis during normal infection.

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